THE EFFECT OF GLUCOSE ON THE GROWTH OF FILAMENTOUS FUNGI IN JET FUEL

CAPT ERICK A. SNELLMAN
JACK E. GREATHOUSE

DEPT OF BIOLOGY
USAF ACADEMY, COLORADO 80840

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Captain Erick A. Snellman
Jack E. Greathouse

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Department of Biology
USAF Academy Colorado 80840

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This research report entitled "The Effect of Glucose on the Growth of Filamentous Fungi in Jet Fuel" is presented as a competent treatment of the subject, worthy of publication. The United States Air Force Academy vouches for the quality of the research, without necessarily endorsing the opinions and conclusions of the author.

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DONALD R. ERBSCHLOE, Lt Col, USAF
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The Effect of Glucose on the Growth of Filamentous Fungi in Jet Fuel

Running Title: Growth of Fungi in Jet Fuel with Glucose

ERICK A. SNELLMAN[1], JACK E. GREATHOUSE, MILES J. PRICE, TONI TERHUNE, TIFFANY J. REITNAUER, COURTNEY L. MINER, HEIDI C. BERTRAM, LISA M. LAU, AND KELLY ANN COSTIGAN

Department of Biology, Suite 2A29, 2354 Fairchild Dr., United States Air Force Academy, Colorado Springs, Colorado 80840

[1] *2nd Airlift Squadron, 3503 Surveyor Street, Pope AFB, North Carolina 28308*

ABSTRACT

Filamentous fungi were isolated from jet fuel-contaminated soils subjected to small, repeated spills. The fungi were tested for their ability to grow in 1, 2, and 5% jet fuel (JP-4) and the same concentrations supplemented with 1% glucose. Growth (reported as dry weight) in fuel and fuel and glucose mixtures were compared to growth in 1% glucose. Significantly more growth by all isolates occurred in fuel and glucose mixtures than glucose alone. A single strain of *Penicillium* produced the best growth under all conditions and was used in experiments analyzing hydrocarbon degradation using gas chromatography/mass spectrometry (GC/MS). *Penicillium* was grown in 2 and 3% fuel alone and with supplemental glucose (1%) and the non-polar extracts examined to assess hydrocarbon degradation at 7 and 14 days. The addition of glucose to the culture medium significantly accelerated fuel metabolism within 7 days. No detectable jet fuel hydrocarbons were present in extracts from cultures harvested at 14 days with or without supplemental glucose. We suggest the addition of glucose may enhance the growth of filamentous fungi in contaminated soils as well as accelerate bioremediation of fuel hydrocarbons and should be considered as an addition to other bioremediation treatments.

INTRODUCTION

The United States Air Force, other DoD agencies, and commercial industries store large quantities of jet fuel and other petroleum products. Even in circumstances where the processing, use, and storage of these products are highly regulated and closely monitored, hydrocarbon contamination of soil and ground-water is a potential problem. Bioremediation is a promising and cost effective alternative to costly physical removal. As such, the search for optimum conditions which increase the rate and quantity of hydrocarbon removal from contaminated sites is continuous.

Many microorganisms have been identified which are able to metabolize petroleum hydrocarbons (2) and recent emphasis has been placed on understanding the conditions which enhance bioremediation irrespective of the microorganisms involved. Numerous workers have investigated the effects of temperature, nutrient content, oxygen concentration, moisture and pH, singularly or in combination (5, 9, 10, 12, 13, 17). Hinchee and Arthur (10) found that forced aeration, coupled with the addition of nutrients and moisture, stimulates hydrocarbon-degrading microorganisms while Song *et al.* (17) reported that maximal bioremediation of many petroleum products occurs at 27^{o} C. In studying the effect of pH, Casarini *et al.* (5) demonstrated landfarming systems with pH control (pH 7.0) are more effective in the biodegradation of oily sludges than systems without pH control. Few studies, however, have addressed those factors that enhance the growth and assimilation of hydrocarbons by filamentous fungi although the ability of these organisms to degrade petroleum products is well documented (6, 11, 14, 19). Filamentous fungi are strong candidates for bioremediation since their capacity to degrade petroleum hydrocarbons has been shown to be as high as hydrocarbon-degrading bacteria (15). In this study, we have investigated the effect of glucose on hydrocarbon assimilation by filamentous fungi isolated from a contaminated soil subjected to repeated jet fuel spills.

MATERIALS AND METHODS

Isolation of Soil Fungi

Soil samples were aseptically collected from the United States Air Force Academy fuel pit area and stored at 5°C. Jet fuel (grade JP-4) used in all experiments was collected in sterile glass jars from the airfield fuel storage tank and stored at 5°C. Fungi were isolated from soil grains placed on Bushnell Haas (BH) medium (4)(per liter: 0.02g $CaCl_2$; 0.2g $MgSO_4$; 1.0g KH_2PO_4; 1.0g K_2HPO_4; 1.0g NH_4NO_3; 0.1ml saturated solution $FeCl_3$; solidified with Bacto agar (15.0g) and containing 1% fuel as sole carbon source (v/v)). Isolates of individual fungi were grown in pure culture. Identification of isolates to genus was accomplished using light microscopy following keys of Barnett and Hunter (3) and von Arx (20). Isolates were maintained on modified BH slants augmented with 1% glucose (w/v), grown at 25° C for 72h and stored at 5°C.

Inoculum Preparation

The fungal inoculum was prepared by transferring small pieces of mycelium from stock cultures to potato dextrose agar and growing for 72h at room temperature (24°C). One square centimeter of medium that was heavily overgrown with mycelia was transferred to 125 ml of nutrient broth in a 250 ml Erlenmeyer flask and incubated at room temperature on a Thermolyne rotary shaker at 125 rpm for 72h. The nutrient broth was decanted and discarded and the mycelia were transferred to a sterile Monel metal blender. Thirty milliliters of sterile deionized water was added to the blender and the mixture macerated for one minute, then transferred to a sterile 250 ml Erlenmeyer flask. The dry weight of each inoculum was estimated by filtering 1.0 ml aliquots (three replicates) through a Millipore filter apparatus and Whatman glass microfibre filters (1.5μm retention) under vacuum. Filter papers were dried for 72h at 80° C and then weighed using a Mettler analytical balance to obtain mean inoculum dry weight.

Growth of Fungi with JP-4 and Dry Weight Analysis

Growth flasks were prepared by placing BH medium (with 1% glucose (w/v) if required) in 125 ml Erlenmeyer flasks and autoclaving at 121 psi for 20 minutes. Following sterilization, JP-4 fuel was added by cold-filter sterilization using a 20 ml plastic syringe and a Vanex 0.45*um* hydrophilic nylon membrane filter unit. Culture conditions were as follows(w/v): 1% glucose; 1% fuel; 1% fuel, 1% glucose; 2% fuel; 2% fuel, 1% glucose; 5% fuel; 5% fuel, 1% glucose in 50 ml BH medium (pH 6.7). One ml inoculum aliquots were aseptically transferred to experimental growth flasks. Inoculated flasks were prepared in triplicate for each of the seven culture conditions and placed on a Thermolyne rotary shaker and shaken at 125 rpm for 15 days.

At the conclusion of the incubation period, fungi were harvested by filtering the contents of each flask using a Millipore filter apparatus as described for inoculum preparation. Each flask was rinsed with 10 ml deionized water to remove any residual mycelia from the flask and then filtered. Filter papers were dried and weighed as described for dry weight inoculum preparation.

Extraction of JP-4 Hydrocarbons

Experimental and control flasks were prepared and incubated as described above with the following exceptions. All experiments were performed using only the *Penicillium* isolate. All glassware used was acid washed (1% HCl), rinsed in deionized water and dried prior to use. Culture conditions were as follows: 1% glucose; 2% fuel; 2% fuel, 1% glucose; 3% fuel; 3% fuel, 1% glucose. Control flasks contained identical culture media but were not inoculated.

Flasks were prepared and inoculated as described above, then harvested at 7 or 14d using the following procedure. To maximize detection of fuel hydrocarbons, the culture medium from the three replicate flasks for each growth condition was combined by filtering through a Millipore filter and the filtrate collected in a 1000 ml Erlenmeyer flask. Two milliliters of dichloromethane (HPLC-GC/MS

grade) were added to each culture flask and the flask agitated to remove any remaining fuel. The residual dichloromethane and fuel mixture was also filtered and the filtrate collected. A final wash of the Millipore glassware and mycelia was performed by rinsing with 2 ml of dichloromethane. The filtrate was poured into a 250 ml glass separatory funnel followed by a final addition of 2 ml dichloromethane and shaken for approximately 5 minutes. After separation, both the non-polar and polar phases were collected in small glass vials for gas chromatography/mass spectroscopy and glucose assay, respectively.

Analysis of JP-4 Hydrocarbons

The non-polar samples were analyzed on a Hewlett Packard GC/MS (model 5985B) with a 30m glass capillary column (DB-1, J&W Scientific, Inc.) using helium as the carrier gas at a flow rate of 3 ml min^{-1}. Samples (0.1*ul*) were injected at a port temperature of 250^{o}C and constant column temperature of 50^{o}C for 10 minutes followed by an increase to 250^{o}C at a rate of 2^{o}C min^{-1}. The polar phase was tested for the presence of glucose using Boehringer Mannheim diagnostic strips.

RESULTS

Compared to other isolates, *Penicillium* sp. produced the most biomass when jet fuel was the sole carbon source or when supplemented with glucose (Table 1). Although there is no significant difference between mean dry weights of *Penicillium* grown in different concentrations of jet fuel (1, 2, or 5%) with 1% glucose, the dry weights obtained in these cultures are significantly greater than the mean biomass produced when this isolate is grown in 1% glucose alone ($p < 0.05$, two-way ANOVA test (18)). *Fusarium* (isolate 3J) also showed significantly greater growth in fuel and glucose mixtures than obtained in 1% glucose alone. However, it can be seen that growth of this isolate is inhibited in the treatment containing 5% fuel, 1% glucose with respect to other fuel treatments (1% & 2% fuel) with 1% glucose. Results shown for *Fusarium* (isolate 3L) and *Trichoderma* are similar; growth in 1% fuel, 1% glucose and 2% fuel, 1% glucose was significantly greater than 1% glucose alone. However, mean dry weight produced in 5% fuel, 1% glucose is not significantly different from

1% glucose, again suggesting growth inhibition at higher fuel concentrations. Of particular interest is the fact that more biomass is produced in 1% fuel, 1% glucose (mean = 141.9 mg) and 2% fuel, 1% glucose (mean = 143.1 mg) than in 1% glucose alone (mean = 118.7 mg) for all four isolates ($p < 0.05$, df = 168). The dry weights obtained for each isolate when grown in treatments containing only fuel were not significantly different from each other with one exception: *Penicillium* produced more biomass when grown in 1% and 2% fuel than when grown in 5% fuel indicating growth inhibition at the higher fuel concentration.

Gas chromatograms of non-polar extracts from experimental (inoculated) and control (uninoculated) culture flasks containing 2% fuel and 2% fuel, 1% glucose harvested at 7 and 14d are shown (Figs. 1 and 2). Results obtained from cultures containing 3% fuel and 3% fuel, 1% glucose were similar to those shown and are not illustrated. A chromatogram of untreated JP-4 fuel is included (Fig. 1A) for comparison.

There were no significant losses of fuel hydrocarbons in control cultures harvested at 7d compared to uninoculated fuel (Fig. 1). Percent standard areas for predominant peaks (39 - 69 mins retention time (RT)) were calculated for all chromatograms and used in the following comparisons. Removal of fuel hydrocarbons can be seen in 7d cultures containing *Penicillium* but significantly greater quantities are lost in inoculated cultures with glucose added (Fig. 1D and Fig. 1E). No specific hydrocarbon components appear to be selectively degraded since the relative concentration of hydrocarbon components remains similar in all conditions. Analysis of non-polar fractions from treatments containing *Penicillium* grown in 1% glucose alone show no detectable release of hydrocarbons into the medium by the organism (data not shown) suggesting that any changes in hydrocarbon concentrations are due to the removal of hydrocarbons (by degradation or evaporation), not hydrocarbon production by the fungus.

Many of the lower boiling hydrocarbons (RT < 35 mins) were volatilized in the control cultures harvested at 14d but the relative concentrations of residual hydrocarbons remained similar (Fig. 2). Most importantly, no hydrocarbons are detectable by GC/MS in the 2% (Figs. 2C and 2D)and 3% (data not shown) fuel cultures containing *Penicillium* whether glucose is present or not.

In experiments harvested at 7 and 14d, glucose was detected in the aqueous phase using Boehringer Manheim diagnostic strips (data not shown). The greatest quantity of glucose metabolized was in cultures containing 1% glucose and *Penicillium*; all other inoculated and control treatments showed that most of the original glucose remained in the aqueous phase. This indicates the growth of *Penicillium* in treatments containing varying fuel concentrations and glucose is not limited by the quantity of glucose present. Further, it suggests preferential use of fuel hydrocarbons by the fungus.

DISCUSSION

The three genera of fungi isolated in this study have been previously reported to possess hydrocarbon degrading abilities (6, 11, 14). Hydrocarbon-degrading strains of *Penicillium* spp. have been found to be abundant in oil contaminated soils (6, 11, 14). *Fusarium* and *Trichoderma* spp. have also been isolated in similar areas but less frequently (11). The growth results reported here (Table 1) for cultures containing 1, 2, and 5% jet fuel are similar to those values reported previously (19) for filamentous fungi grown in Nos. 2 and 4 fuel oils. However, in this study, significantly greater growth was obtained when glucose was added to the culture medium.

The addition of jet fuel to soils has been found to stimulate fungal growth (16). Bioremediation treatment (tilling, liming and fertilization) has also been shown to enhance the growth of fungi (16) and accelerate the loss of jet fuel from soils (17, 21). This study shows the growth of fungi is also stimulated by the addition of glucose in media containing jet fuel (Table 1). All fungi tested produced more biomass in cultures containing 1% fuel, 1% glucose and 2% fuel, 1% glucose than 1% glucose alone although growth inhibition occurs at higher fuel concentrations in some strains. This suggests that the growth of fungi in hydrocarbon contaminated soils may be limited by the absence of glucose

(or other easily metabolized carbon sources) and that supplemental glucose should be considered in conjunction with other bioremediation treatments such as pH control, aeration, and fertilization. Dean-Ross (7) and Dean-Ross *et al.*, (8) found that evaporation was primarily responsible for removing the low molecular weight hydrocarbons of jet fuel from soils within 5 days. Higher molecular weight components were shown to be less volatile and more likely to be removed by biodegradation (7). Similar evaporative loss of lower boiling hydrocarbons is reported here, but it can be seen that it occurs later, between 7 and 14d (Figs. 1 and 2). Biodegradation contributes significantly to the removal of the remaining high boiling hydrocarbons (RT > 35 mins) as the quantity of these components is greatly reduced in 7d and no detectable hydrocarbons are present at 14d (Figs. 1 and 2). Uninoculated controls show persistence of these same high boiling components (Fig. 2).

The isolate of *Penicillium* has been shown to be capable of removing all JP-4 hydrocarbons in the culture conditions described (Fig. 2C and 2D). This result differs from that reported previously (1) which described biodegradation of JP-4 by an *in situ* microbial community to be compound specific. In cultures containing fuel supplemented with 1% glucose, *Penicillium* degrades a significantly greater quantity of hydrocarbons in 7d compared to cultures that contain only fuel. This shows that glucose increased the rate of hydrocarbon metabolism (Fig. 1). After 14d, non-polar extracts contain no detectable hydrocarbons regardless of the presence of glucose in the medium. We have considered the fact that much more biomass is produced by the fungus when grown in fuel supplemented with glucose than fuel alone (Table 1) but in either condition, complete degradation of hydrocarbons occurs (Figs. 2C and 2D). We suggest the addition of glucose may be useful in increasing fungal growth and the rate of hydrocarbon degradation in fuel contaminated soils. In addition, growth studies using radiocarbon labeled jet fuel may elucidate the metabolic fate of such intrusive hydrocarbons in filamentous fungi.

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FIGURE LEGENDS

FIG. 1. Untreated fuel (A) and non-polar extracts from inoculated flasks containing *Penicillium* or from uninoculated control flasks harvested at 7 days as analyzed by GC/MS. Culture conditions: (B) 2% fuel (uninoculated); (C) 2% fuel, 1% glucose (uninoculated); (D) 2% fuel (inoculated); (E) 2% fuel, 1% glucose (inoculated). **Note:** The GC column was replaced after run (C) with an identical column (DB-1, J & W Scientific) due to a septum bleed identified by mass spec analysis. All equipment parameters were identical (see text); retention times shift in subsequent runs but all predominant peaks were matched to previous runs by mass spec.

FIG. 2. Non-polar extracts from inoculated flasks or uninoculated control flasks harvested at 14 days as analyzed by GC/MS. Culture conditions: (A) 2% fuel (uninoculated); (B) 2% fuel, 1% glucose (uninoculated); (C) 2% fuel (inoculated); (D) 2% fuel, 1% glucose (inoculated).

TABLE 1. Dry weight values of fungi isolated from fuel contaminated soil grown in fuel and fuel/glucose media

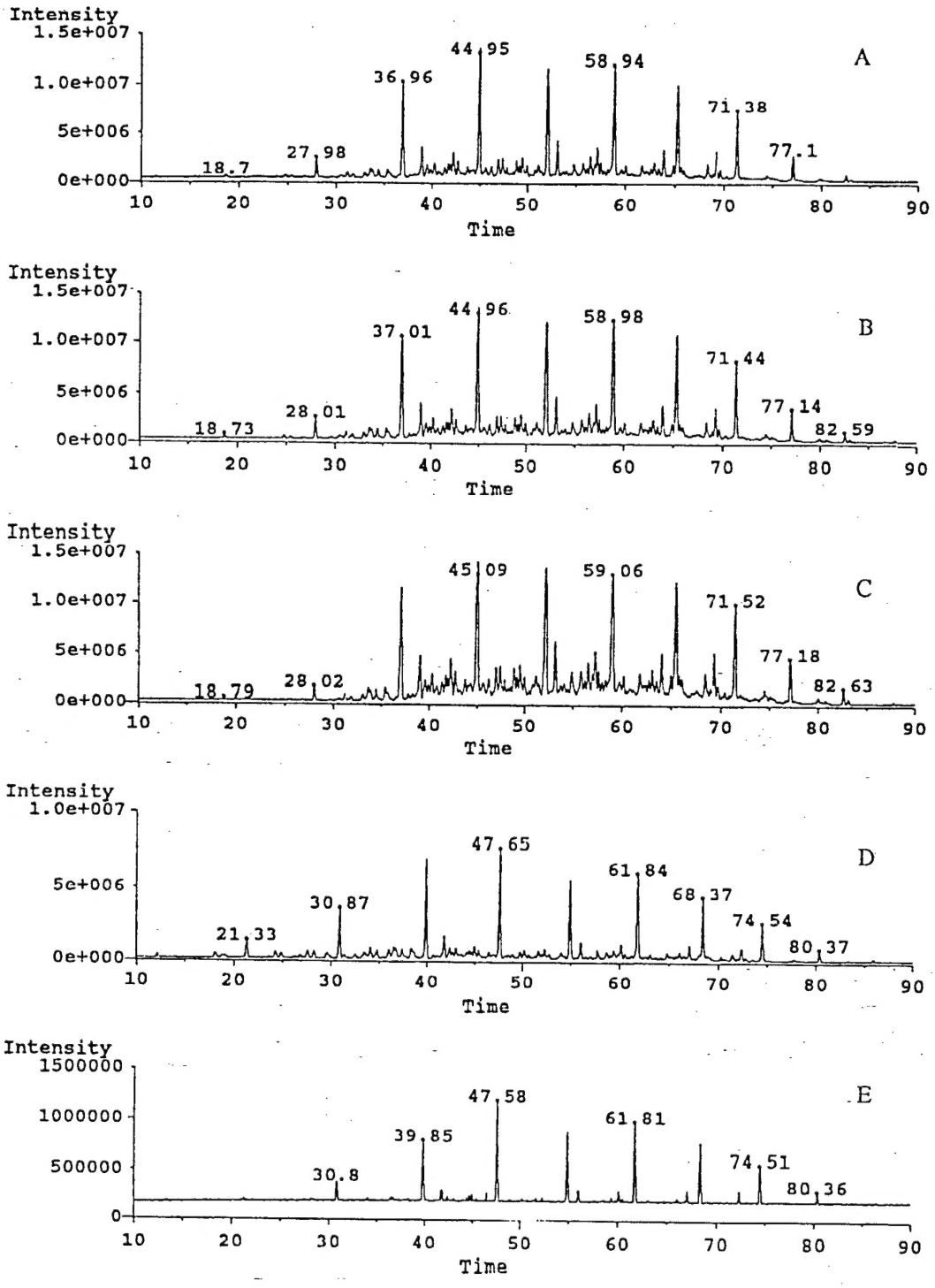
Species isolate (No.) (df [1])	Mean dry weight (mg)/flask ± SD						
	1% glucose	1% fuel 1% glucose	2% fuel 1% glucose	5% fuel 1% glucose	1% fuel	2% fuel	5% fuel
Fusarium sp. (3L) (df=56)	86.5 ± 12.7	102.0 ± 10.0*	108.8 ± 13.7*	92.0 ± 18.7†	11.0 ± 9.3	3.7 ± 1.5	11.5 ± 7.9
Fusarium sp. (3J) (df=42)	83.3 ± 25.8	106.2 ± 23.7*	101.2 ± 19.5*	92.3 ± 13.4*†	5.1 ± 4.4	8.8 ± 6.6	2.1 ± 1.9
Trichoderma sp. (2I) (df=42)	148.5 ± 19.8	171.7 ± 29.3*	170.7 ± 35.8*	160.5 ± 37.8†	4.2 ± 1.9	3.0 ± 2.2	3.4 ± 1.6
Penicillium sp. (3K) (df=42)	156.3 ± 11.7	187.5 ± 10.0*	191.7 ± 9.8*	194.6 ± 18.8*	11.9 ± 3.5	12.0 ± 4.4	3.7 ± 2.0

All treatments with glucose added are significantly different from treatments with fuel alone (two-way ANOVA test, $p < 0.05$)

[1]degrees of freedom

* Significantly different from 1% glucose ($p < 0.05$)

† Significantly different from 1% fuel, 1% glucose ($p < 0.05$)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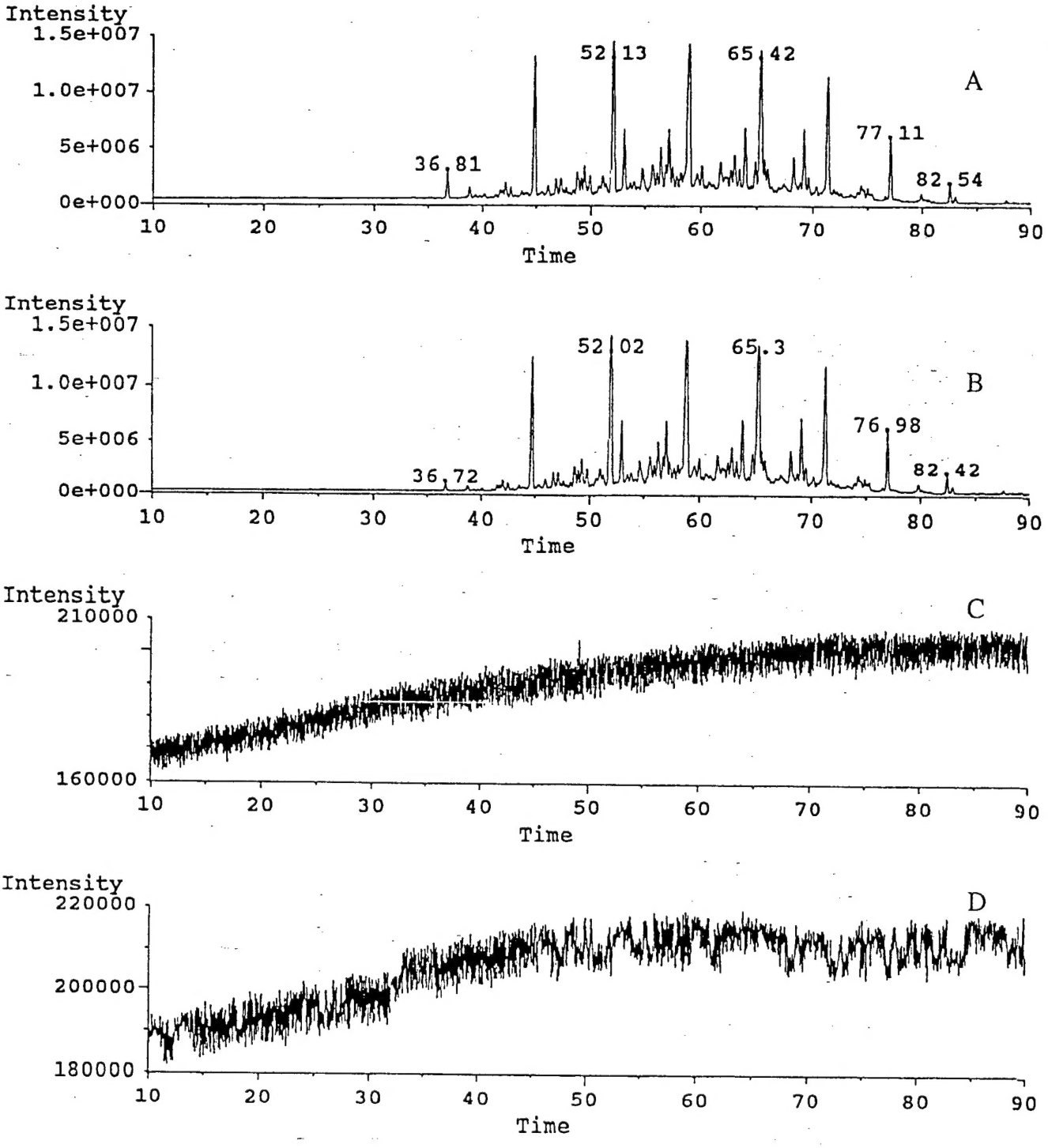
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